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RESEARCH ARTICLE

Permeability of postzygotic barriers: embryology of a partially fertile *Epidendrum* (Orchidaceae) hybrid

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Keywords

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ABSTRACT

- Hybrid zones offer unique insight into reproductive barriers and plant speciation mechanisms. This study investigated postzygotic reproductive isolation in the natural hybrid *Epidendrum* × *purpureum*, which occurs in sympatry with its parent species, *Epidendrum denticulatum* and *E. orchidiflorum*.
- We examined the development of male and female gametophytes and the events leading to seed formation in this hybrid zone. Floral buds and flowers from *E.* × *purpureum* individuals were collected at various stages of development. Both self-pollination and backcrosses between hybrids and parental species were performed to follow ovule and seed development up to 60 days after pollination. The material was analysed using optical and confocal microscopy.
- In most hybrids, microsporogenesis and microgametogenesis occur regularly, forming viable male gametophytes. Non-viable male gametophytes were also observed and are the result of symmetrical mitotic division. The development of the female gametophyte occurs after self-pollination, and proceeds regularly, resulting in a reduced female gametophyte. Embryo development in the parental species occurs without abnormalities, while in backcrosses between hybrids and parental species, most embryos degenerate.
- Embryo degeneration in the crosses between hybrids can be explained by genetic incompatibilities. The co-occurrence of viable embryos and degenerating embryos in backcrosses between hybrids and parental species point to incomplete postzygotic reproductive barriers between the hybrid and the progenitors. Our findings suggest that *E.* × *purpureum* could facilitate gene flow between parental species, as much of its embryological development occurs without abnormalities.

INTRODUCTION

Processes related to the origin and maintenance of species have attracted the attention of evolutionary biologists for centuries (Darwin 1859; Coyne & Orr 2004; Baack *et al.* 2015). Species are fundamental units of biology; however, defining and delimiting species is challenging (de Queiroz 2007). Various concepts have been used to define species, including the concept of biological species, which relates the process of speciation to the evolution of reproductive isolation barriers, resulting in the interruption or reduction of gene flow between divergent lineages (Dobzhansky 1937; Mayr 1942; Coyne & Orr 2004).

Reproductive barriers in plants are classified as pre- and post-pollination. Pre-pollination barriers are exclusively prezygotic and include ecological and reproductive factors, such as geographical isolation, asynchronous flowering, and isolation from pollinators (Sobel *et al.* 2010). Post-pollination barriers can be prezygotic, such as pollen–stigma or pollen–stigma incompatibility, during signalling and/or fusion between female and male gametes (Moyle *et al.* 2014) or postzygotic, involving sterility, non-viability, or hybrid necrosis (Baack *et al.* 2015). Pre- and postzygotic barriers can differ in the intensity of their effects, and in many species, reproductive isolation is not caused by one isolating factor alone, but by complex interactions between different types of reproductive barriers (Widmer *et al.* 2009).

Studies on hybrid zones are essential for understanding the evolution of reproductive isolation in plants, as they enable us to recognize how the individual components of reproductive isolation prevent interspecific gene flow and maintain the integrity of the parental species (Abbott 2017). Hybrid zones in Orchidaceae present great complexity, with species exhibiting a strong pre-pollination isolation mechanism (Xu *et al.* 2011; Whitehead & Peakall 2014), prezygotic post-pollination at the level of pollen–stigma interaction (Pellegrino *et al.* 2010), strong postzygotic barriers, presenting hybrids with high embryo mortality and sterility (Cozzolino & Scopece 2008; Scopece *et al.* 2008; Pinheiro *et al.* 2010, 2015), and zones showing reproductive isolation resulting from the combination of pre- and post-pollination reproductive barriers (Scopece *et al.* 2013; Tao *et al.* 2018; Arida *et al.* 2021; Zhang *et al.* 2022).

Information on speciation in the natural hybrid zones of Orchidaceae in the Neotropical regions is restricted to a few genera, such as *Cypripedium* L. (Szlachetko *et al.* 2017),

Fig. 1. Parental species and hybrid in the Restinga de Massambaba. (a) *Epidendrum denticulatum*. (b) *Epidendum orchidiflorum*. (c) *Epidendrum* × *purpureum*.

Bulbophyllum Thouars (Borba & Semir 1998; Azevedo *et al.* 2006; Fiorini *et al.* 2023), and *Epidendrum* L. (Pinheiro *et al.* 2010, 2015, 2016; Moraes *et al.* 2013; Vega *et al.* 2013; Marques *et al.* 2014; Arida *et al.* 2021). *Epidendrum* is one of the largest orchid genera in Neotropical regions, showing wide morphological variation, and has therefore been used as a model system to investigate the evolution of reproductive isolation in orchids (Hágsater & Soto-Arenas 2005; Pinheiro & Cozzolino 2013). *Epidendrum* hybrid zones present complex reproductive barriers, with species showing incomplete reproductive isolation between the pairs studied, with variation in interspecific gene flow and introgression (Pinheiro *et al.* 2010; Moraes *et al.* 2013; Vega *et al.* 2013; Marques *et al.* 2014), to high levels of hybrid sterility (Pinheiro *et al.* 2015, 2016; Arida *et al.* 2021). The ploidy and genetic structure of the hybrid and parental lineages in the different hybrid zones of the genus have been well characterized. However, the mechanisms that lead to the formation of hybrid specimens with different levels of sterility remain unknown.

In this study, we investigated the reproductive development pattern of *Epidendrum* × *purpureum* hybrid specimens, which resulted from natural crosses between *Epidendrum denticulatum* Barb. Rodr. (Fig. 1a), and *Epidendrum orchidiflorum* Salzm (Fig. 1b) (Rodrigues 1877; Arida *et al.* 2021). The hybrid *E.* × *purpureum* (Fig. 1c) has a chromosome number ranging from 2n = 104 to 2n = 106, and lower reproductive success than the parental species, which is probably related to the size of the genome and the abnormalities that occur during meiosis (Arida *et al.* 2021). Experimental crosses have found the presence of seeds with varying levels of fertility and a significant decrease in seed viability in crosses between hybrids and backcrosses, where hybrids acted as pollen recipients and donors for the parental species (Arida *et al.* 2021), indicating the potential presence of genetic incompatibilities. These incompatibilities can influence the development of the male and female gametes of hybrid individuals, which may reduce the viability of progeny from crosses involving hybrid plants, as observed in different studies (Zeng *et al.* 2009; Shin *et al.* 2021; Gautam *et al.* 2023). Therefore, to clarify which mechanisms are responsible for the fertility variation in hybrid specimens of *E.* × *purpureum*, our aim was to answer the following questions: (i) what is the development pattern of the gametophytes and of male and female gametes of *E.* × *purpureum*; (ii) how do *E.* × *purpureum* embryos develop; (iii) which post-pollination events are related to the formation of sterile and fertile seeds in backcrosses between hybrids and parental species?

MATERIAL AND METHODS

Species and sampling sites

Epidendrum denticulatum Barb. Rodr. occurs in the Cerrado and coastal vegetation of southeastern Brazil (Pinheiro *et al.* 2015). *Epidendrum orchidiflorum* Salzm. occurs in dry vegetation communities found in coastal areas and Caatinga vegetation in northeastern Brazil (Pachon 2016). Both species occur over ca. 300 km in southeastern Brazil and overlap in the coastal vegetation of sand dunes along the coast of the state of Rio de Janeiro, where they naturally hybridize, forming the hybrid *Epidendrum* × *purpureum* (Rodrigues 1877; Arida *et al.* 2021). The samples were collected in Restinga de Massambaba (Cabo Frio—RJ), where the parental species and hybrids occur in sympatry, and were cultivated in the Orchidarium of Universidade Estadual de Campinas. Voucher specimens were deposited at the Herbarium of the University of Campinas (HUEC), Campinas, São Paulo, Brazil, under the following record numbers: 212843, 212844, 212845.

Experimental pollination

Self-pollination was performed to analyse the development of the female gametophyte and the events that lead to the development of fertile and sterile seeds in the *E.* × *purpureum* hybrid, as well as the development of female gametophytes and embryos in the parental species *E. denticulatum* and *E. orchidiflorum*. Backcrosses was performed using hybrids as pollen donors and recipients with each parental species to analyse the development of female gametophytes and embryos. Ten pollinations were conducted with five individuals for each type of cross.

Optical microscopy

To investigate the viability of gametophytes and male gametes, 35 anthers from floral buds and flowers in anthesis of the hybrid *E.* × *purpureum* were collected. To analyse female gametophyte

and embryo development, fruits from different experimental pollinations described above were collected at 10, 15, 20, 25, 30, 35, 40, 45, 50, and 60 days after pollination (DAP). The samples were fixed in 10% neutral-buffered formalin (Lillie 1965), dehydrated in a graded ethanol series, and infiltrated with hydroxyethylmethacrylate (Historesin, Leica) (Gerrits & Smid 1983). The samples were sectioned at a thickness of 3 μm on a Leica RM2245 rotary microtome, stained with 0.05% toluidine blue in phosphate buffer pH 4.5 (Sakai 1973), and mounted on Entellan synthetic resin (Merck). The slides were analysed under an Olympus BX51 optical microscope and photographed using an Olympus DP73 digital camera.

Laser scanning confocal microscopy

To reconstruct the male gametophytes, anthers were collected. Thick sections of the samples, obtained using the rotary microtom, e as described above, were analysed using a laser scanning confocal microscope (Leica TCS SPE) with a 405 nm laser channel to excite the following fluorochromes: 0.5% aniline blue (Oparka & Read 1994), emitting at 480–525 nm; 0.01% auramine O (Heslop-Harrison 1977), emitting at 540–656 nm; 0.1% calcofluor white (O'Brien & McCully 1981), emitting at 431–490 nm; and 6-diamidino-2-phenylindole – DAPI (Coleman & Golf 1985), emitting at 408–457 nm to detect callose, phenolic compounds (sporopollenin), cellulose, and nuclear DNA, respectively. Images were captured by direct acquisition with Z-pitch ranging from 0.13 to 0.27 mm, which generated 30 to 40 optical sections in LAS AF Lite 2.6.0 software (Leica Microsystems).

RESULTS

Pollinia development in *E.* × *purpureum*

The anthers of the hybrid *E.* × *purpureum* are bithecal and tetrasporangiate, and each theca has two pollinia and two caudicles (Fig. 2a). The anther wall in young bud stamens consists of an epidermis, endothecium, two middle layers, and biseriate tapetum. The epidermis and endothecium remain uniseriate throughout the development. The tapetum is glandular and presents cells with a dense cytoplasm and evident nuclei (Fig. 2b).

The sporogenous cells (Fig. 2b) differentiate into microspore mother cells (MiMCs) in the young anther (Fig. 2c). We observed the deposition of callose during the differentiation of MiMCs, individualizing the cells. MiMCs undergo the first stage of meiosis, originating a dyad of microspores (Fig. 2e). The cells of the dyad undergo the second stage of meiosis, which is of the successive type, resulting in a tetrad of microspores that remain united and have different conformations (Fig. 2f). The microspores have cell walls with an evident amount of cellulose (Fig. 2g). At the end of the meiotic process, the exine is deposited on the microspores cell walls as well as on the periphery of the pollinia (Fig. 2f,h). After maturation, most of the microspores undergo asymmetrical mitotic division (Fig. 2i,j), which gives rise to the male gametophyte (Fig. 2k,l), which is formed by a larger cell, the vegetative cell, and a smaller cell, the generative cell, which initially has a parietal position (Fig. 2j). Later, the generative cells migrate into the cytoplasm of vegetative cells and start to present a condensed nucleus (Fig. 2k,l).

Some alterations were observed during male gametophyte development in the hybrid specimens. Some microspores underwent a symmetrical mitotic process, resulting in male gametophytes with two identical cells similar to the vegetative cells of functional male gametophytes (Fig. 2k,m). Occasionally, male gametophytes were observed only in the cell walls, without any cytoplasmic content (Fig. 2m). Of the 35 anthers analysed, only one showed bicellular male gametophytes with degeneration of the vegetative and generative cells (Fig. 2n), and the other showed total degeneration of the male gametophytes (Fig. 2o).

Caudicle development occurs simultaneously with that of pollinia (Fig. S1) and is visible in the first stages of anther development. Initially, the caudicle is represented by a mass of meristematic cells near the epidermis of the microsporangium (Fig. S1a). Some of these cells increase in size (Fig. S1b) and cellulose is evident in the cell wall (Fig. S1c), whereas the rest remain undifferentiated, with characteristics similar to those of pollinia tapetum (Fig. S1b). While sporogenesis occurs in the pollinia, changes in the caudicle cells were observed (Fig. S1d). The cells that increased in size undergo a meiotic cycle (Fig. S1e,f), resulting in a tetrad of cells similar to that observed in pollinia (Fig. S1g). The cells of the tetrad undergo asymmetrical mitotic division, similar to that observed during the formation of male gametophytes of the pollen (Fig. S1h). At this stage, it is possible to observe the deposition of the exine on the cell wall of the caudicle cells (Fig. S1i).

Experimental pollination: female gametophyte development

The development stages of the different types of pollination are summarized in Fig. 3. The stages of development of the female gametophyte resulting from intraspecific and interspecific experimental pollination are described jointly because of the similarity of the events (Fig. 4, Figs. S2–S5).

All analysed specimens have flowers at anthesis with undifferentiated ovaries (Fig. 4a, Fig. S3a). Placental proliferation occurs after pollination due to intense mitotic activity. The ovary has three carpels divided into six valves, three fertile with the placental region and three sterile (Fig. 4b, Figs. S3b–S5a), at approximately 10 DAP. At this stage, it is possible to observe ovule primordium in *E.* × *purpureum* and in the parental species *E. denticulatum* and *E. orchidiflorum* (Fig. 4c, Figs. S4b and S5b). Differentiation of ovules is not synchronous, not even within the same fruit, in both the hybrid and parental species.

From 10 to 15 DAP, a cell in the subepidermal layer in ovule primordia differentiates into an archesporial cell (Fig. 4d,e, Figs. S2b–S5c), and cell division in the dermal layer leads to the formation of inner and outer integuments (Fig. 4d–f, Figs. S2c–S5d). The nucellar epidermis has only one cell layer, characterizing the ovule as tenuinucellate (Fig. 4f, Figs. S4d and S5d). From 20 to 30 DAP, the archesporial cell give rise directly to the megaspore mother cell (MMC), which increases in volume and has an evident nucleus (Fig. 4f, Figs. S2c–S5d). At this stage of development, the outer and inner integuments elongate to form the micropyle region (Fig. 4f, Fig. S5d). MMC undergoes the first stage of meiosis, giving rise to a dyad of megaspores (Fig. 4g, Figs. S2d–S5e). In some ovules resulting from the self-pollination treatment in hybrids, a dyad of degenerating megaspores was observed (Fig. 4h). The chalazal megaspore undergoes a second stage of meiosis, which originates a

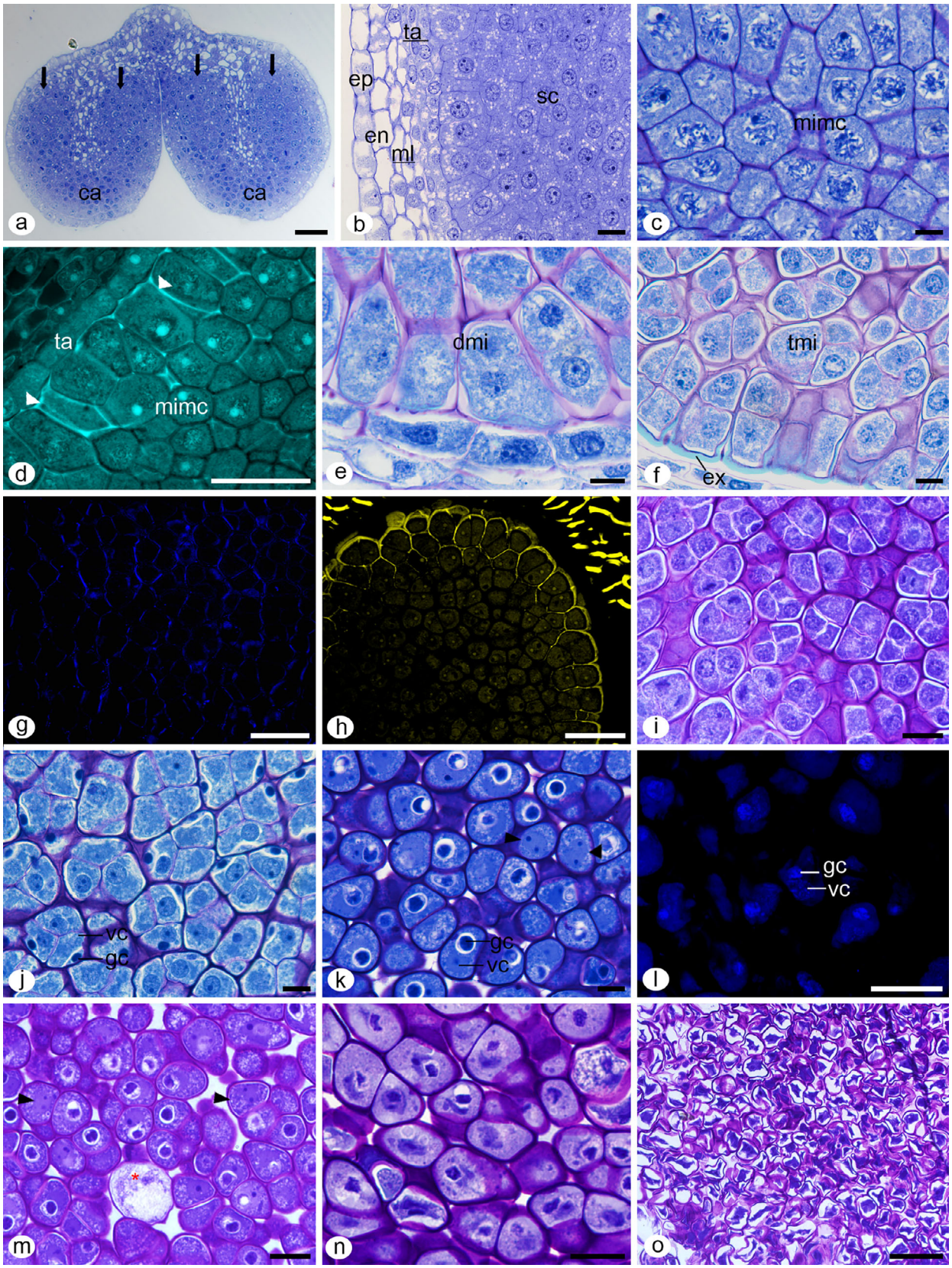

Fig. 2. Pollinia development, microsporogenesis and microgametogenesis in *Epidendrum* × *purpureum*. (a) Overview of the bitecae anther. Black arrows indicate the microsporangia. (b) Anther wall formed by epidermis, endothecium, middle layers and tapetum. The pollinia present sporogenous cells. (c) Microspore mother cells. (d) Callose deposition in the cell wall of microspore mother cell evidenced by aniline blue. (e) Dyad of microspores. (f) Tetrad of microspores. (g) Cellulose in microspore cell wall, evidenced by calcofluor. (h) Unequal deposition of sporopollenin on the male gametophyte wall, evidenced by auramine O. (i) Microspores in mitotic cycle; (j, k) Male gametophyte with vegetative cell and generative cell. Arrowhead indicates male gametophytes that have undergone symmetrical mitotic division. (l) Vegetative cell nucleus and generative cell nucleus, evidenced by DAPI. (m) Pollinia showing viable and non-viable male gametophytes. Arrowhead indicates male gametophytes that have undergone symmetrical mitotic division. Red asterisk indicates male gametophyte without cytoplasmic content. (n) Male gametophytes showing a generative cell and a vegetative cell in degeneration. (o) Degenerate pollinia. (d, g, h, l) Laser scanning confocal microscopy. ca, caudicle; dmi, dyads of microspores; ep, epidermis; en, endothecium; ex, exine; gc, generative cell; ml, middle layer; mimc, microspore mother cell; sc, sporogenic cells; ta, tapetum; tmi, tetrad of microspores; vc, vegetative cell. Scale bars: a, d, o = 50 μm; b, g, h, i, m, n = 20 μm; c, e, f, j, k = 10 μm.

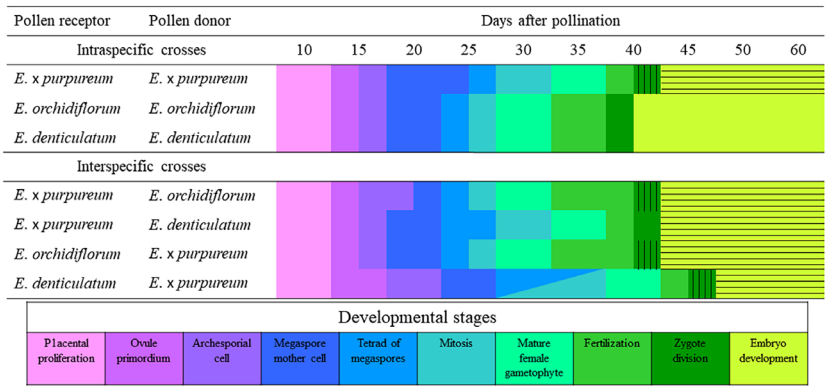

Fig. 3. Development stages of ovule and embryos in the different experimental crosses. Vertical lines refer to stages where zygote division and zygote degeneration were observed at the same time. Horizontal lines refer to stages where embryo development and embryo degeneration were observed at the same time.

triad of megaspores (Fig. 4i, Figs. S2e–S5f). The chalazal megaspore become functional, whereas the other megaspores degenerate (Fig. 4i, Figs. S2e–S5f).

Megagametogenesis begins with the first mitotic division of the functional megaspore, and the two nuclei formed migrate to the micropylar and chalazal poles of the developing female gametophyte due to the formation of a large central vacuole (Fig. 4j, Figs. S2f–S5g). These two nuclei undergo a second mitotic division, leading to the formation of four nuclei (Fig. 4k, Figs. S2g–S5h). These four nuclei undergo the third and final mitotic cycles, resulting in a female gametophyte with eight nuclei. During female gametophyte cellularization, the two synergids and egg cell are organized at the micropylar pole to form the egg cell apparatus, most often in a triangular arrangement (Fig. 4l, Figs. S2h–S5i). Two nuclei migrate to the center of the female gametophyte and are identified as polar nuclei. The three remaining nuclei in the chalazal portion form antipodes (Fig. 4l, Figs. S2h and S4i).

Occasionally, more than one female gametophyte was present in the same ovule (i.e. multiple archesporia). Up to four female gametophytes undergoing mitotic divisions were observed in the same ovule in fruits from intraspecific pollination of the hybrid *E.* × *purpureum* (Fig. 4m,n), as well as in female gametophytes with two egg cells (Fig. 4o). In the interspecific pollinations between *E. orchidiflorum* (pollen recipient) and *E.* × *purpureum* (pollen donor), an ovule with two female gametophytes was observed (Fig. S3i).

Embryo development

The development of embryos resulting from intraspecific and interspecific pollination processes are jointly described. The embryogenesis stages for each pollination type are summarized in Fig. 3.

Fertilization occurred about 30–35 days after intraspecific and interspecific pollination, as evidenced by the penetration of the synergid, which has dense cytoplasm, and the formation of a zygote (Fig. 5a,b, Figs. S2i–S5i). Fusion was observed between polar nuclei and gametic nucleus, forming the primary nucleus of the endosperm, that degenerates (Fig. 5b,c, Figs. S2j, k and S5j,k). The first division of the zygote forms two cells: an apical cell that forms the embryo, and a basal cell that forms the suspensor (Fig. 5b, Fig. S5j). The cells of the inner integument of the seed coat in formation begin to have dense cytoplasm (Fig. 5c), whereas the outer integument elongates.

Developing embryos resulting from the intraspecific pollination of the hybrid specimens (*E.* × *purpureum* × *E.* × *purpureum*) were observed at approximately 40 DAP. In the same fruit, some of the embryos develop without abnormalities, with the apical cells undergoing transverse and longitudinal divisions, which results in the formation of a viable embryo, whereas some of the embryos degenerate (Fig. 5b–k). Degeneration occurs at different stages of development, shortly after the formation of the zygote (Fig. 5e) or after the divisions that lead to the formation of the embryo (Fig. 5f,i,j). Viable

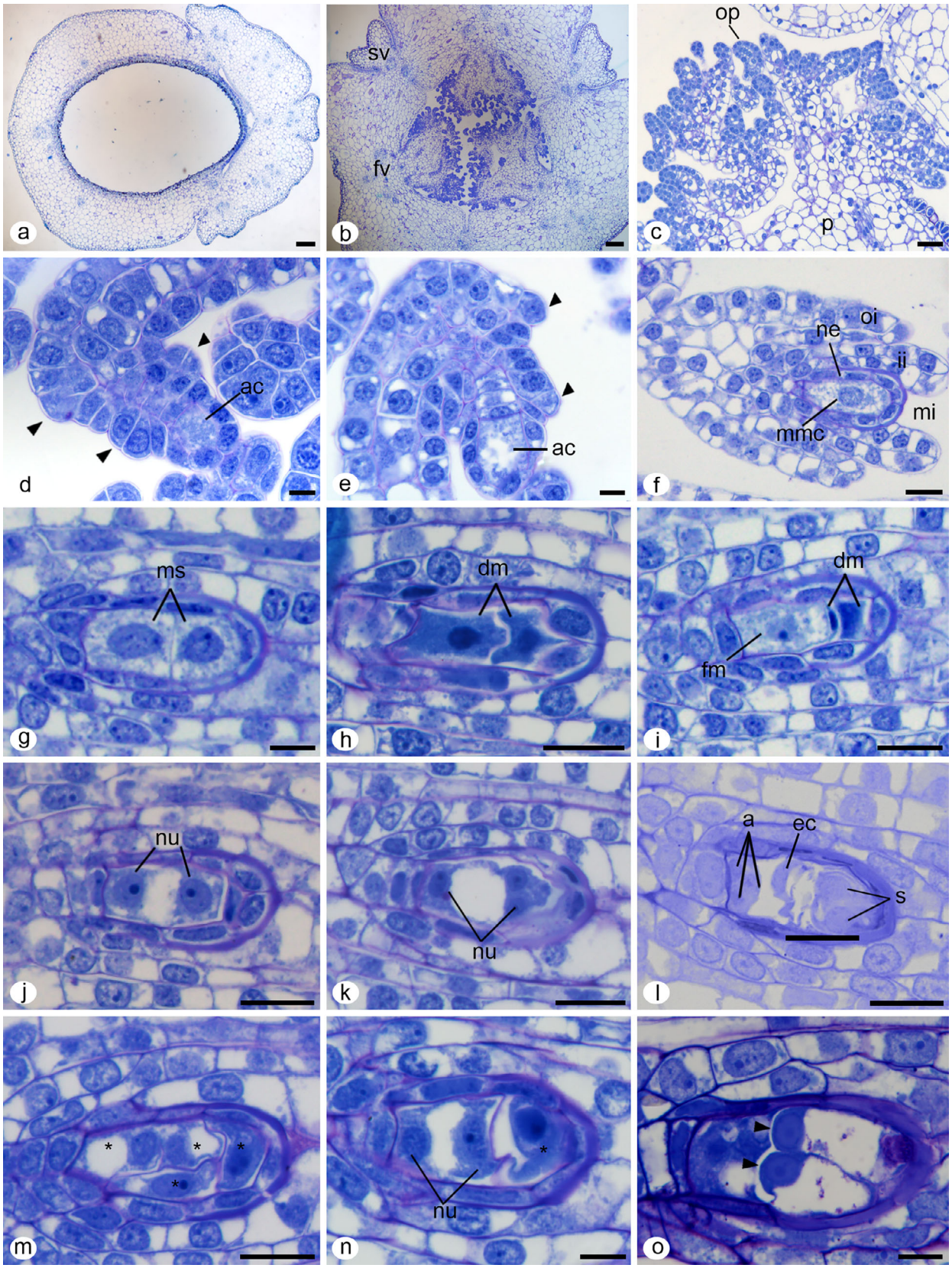

Fig. 4. Ovule development, megasporogenesis and megagametogenesis of *Epidendrum* × *purpureum*. (a) Undifferentiated ovary of the flower at anthesis. (b) Overview of the ovary showing sterile valve and fertile valve. (c) Detail of placental region showing ovule primordium. (d, e) Differentiation of the initial archesporial cell and formation of the integuments, indicated by arrowhead. (f) Megaspore mother cell. (g) Megaspore dyad. (h) Megaspore dyad degenerating. (i) Functional chalazal megaspore. (j) Binucleated female gametophyte. (k). Tetranucleated female gametophyte with three visible nuclei. (l). Female gametophyte with synergids, egg cell, and three antipodes. (m) Ovule with four female gametophytes in development. (n) Ovule with two female gametophytes in development. (o) Female gametophyte with two egg cells indicated by arrowhead. a, antipodes; ac, archesporial cell; dm, degenerating megaspores, ec, egg cell; fm, functional megaspores; fv, fertile valve; ii, inner integument; mmc, megaspore mother cell; mi, micropyle; ms, megaspores; ne, nucellar epidermis; nu, nucleus; oi, outer integument; op, ovule primordium; p, placental region; s, synergids; sv, sterile valve. Scale bars: a, b = 200 μm; c = 50 μm; d, e, h = 10 μm; f, g, l, j, k = 20 μm.

embryos with a morphology similar to that of a globular embryo and a suspensor with elongated cells (Fig. 5g) were observed at 50 and 60 DAP; however, embryos with an irregular morphology were also observed (Fig. 5h). As the embryo grows, the inner integument is crushed, and the outer integument originates the seed coat (Fig. 5g,h). Most of the embryos observed at 50–60 DAP degenerate. Seed coat development in degenerated embryos occurs in a manner similar to that observed in seeds with viable embryos (Fig. 5i,j). Eventually, seeds with two embryos were observed (Fig. 5k).

Embryos resulting from intraspecific pollination of the parental species (*E. denticulatum* × *E. denticulatum* and *E. orchidiflorum* × *E. orchidiflorum*) are viable and do not show any abnormalities during development (Figs. S4k–n and S5k–n). The first division of the zygote forms two cells: an apical cell that forms the embryo, and a basal cell that forms the suspensor (Figs. S4k–n and S5k–n).

In contrast, embryos from backcrosses between hybrids and parental species may be viable or show degeneration (Figs. S2i–o and S3l–o). At approximately 50 DAP, viable embryos present a morphology similar to that of the globular stage and a suspensor with elongated cells (Fig. S3m). At approximately 60 DAP, the numbers of degenerating embryos increases (Fig. S3n–o), and are observed in greater numbers when the hybrid donates pollen to the parental species.

DISCUSSION

Hybrid incompatibility is generally viewed as arising from detrimental interactions between alleles. These alleles, although benign within the parental genomes, become disadvantageous when combined in a hybrid offspring (Coyne & Orr 2004). This model has gained significant support through a growing body of evidence accumulated in recent years. Nevertheless, embryological details of the development stages affected by such genetic incompatibilities are almost absent in the literature. Genetic incompatibilities that cause problems in endosperm development are considered important postzygotic barriers in plants and are the most studied subjects in the context of plant embryology (reviewed by Köhler *et al.* 2021). However, such studies have not focused on orchids because the endosperm tissue is almost absent in this plant family (reviewed by Yeung 2017). Thus, the embryological steps that play a role in hybrid plant viability, especially in orchids, are poorly understood. This study describes, for the first time, the mechanisms involved in the formation of male and female gametes as well as the embryology of a natural hybrid of Orchidaceae. The results demonstrate that most hybrids present viable gametophytes and gametes. However, after fertilization, most embryos degenerate, possibly due to genetic incompatibilities. In backcrosses between hybrids and parental species, there is no incompatibility between pollen and stigma, resulting in regular pollen tube growth, fertilization, with the development of both viable and degenerating embryos. Our results shed light on the specific embryological steps in which genetic incompatibilities may act, decreasing hybrid viability and potentially acting as an important postzygotic barrier.

Pollinia development in *E.* × *purpureum*

The development of male gametophytes in angiosperms is a complex process that requires the coordinated activity of gametophytic and sporophytic tissues controlled by an extensive gene network (Hafidh *et al.* 2016). Male sterility in hybrids can be caused by genetic incompatibilities that affect the formation of gametophytes or surrounding sporophytic tissue (Fishman & Sweigart 2018). In the natural *E.* × *purpureum* hybrid, the relation between gametophytic and sporophytic tissues appeared to occur in a regular manner. No changes were observed in the structure of the anther wall; however, changes were observed in the formation of male gametophytes. The anther wall is formed by a unistratified epidermis and endothecium, a middle layer, and a tapetum with two layers each, similar to that described for the parental species (Alves *et al.* 2024), and other Orchidaceae species (Swamy 1949; Sood & Rao 1988; Kant & Goel 2013; Ghimire *et al.* 2020). No abnormalities were found in the tapetum of the analysed anthers, indicating that the functions performed by this parietal layer, such as nutrition of microspores and secretion of callose and sporoderm (Bhandari 1984), occur regularly.

Although cytogenetic analyses showed only 55.8% meiotic normality in *E.* × *purpureum* (Arida *et al.* 2021), the formation of microspore dyads and tetrads occurs without abnormalities and is similar to that observed in the parental species (Alves *et al.* 2024). The sporogenic tissue does not show any alterations and differentiates into a microspore mother cell that undergoes meiosis, giving rise to microspore tetrads that remain united. This result differs from those observed in commercial hybrids and cultivars of genera from different families, such as *Allium* L. (Alliaceae), *Brassica* L. (Brassicaceae), and *Avena* L. (Poaceae). Cases of male sterility have been reported for these hybrids and can be expressed in different ways, such as underdeveloped male reproductive organs, defects in anther dehiscence leading to non-dispersal of functional pollen, and degeneration of the cytoplasm of microspore mother cells resulting in inability to produce functional pollen (Mayer *et al.* 2013; Shin *et al.* 2021; Zhang *et al.* 2021; Gautam *et al.* 2023).

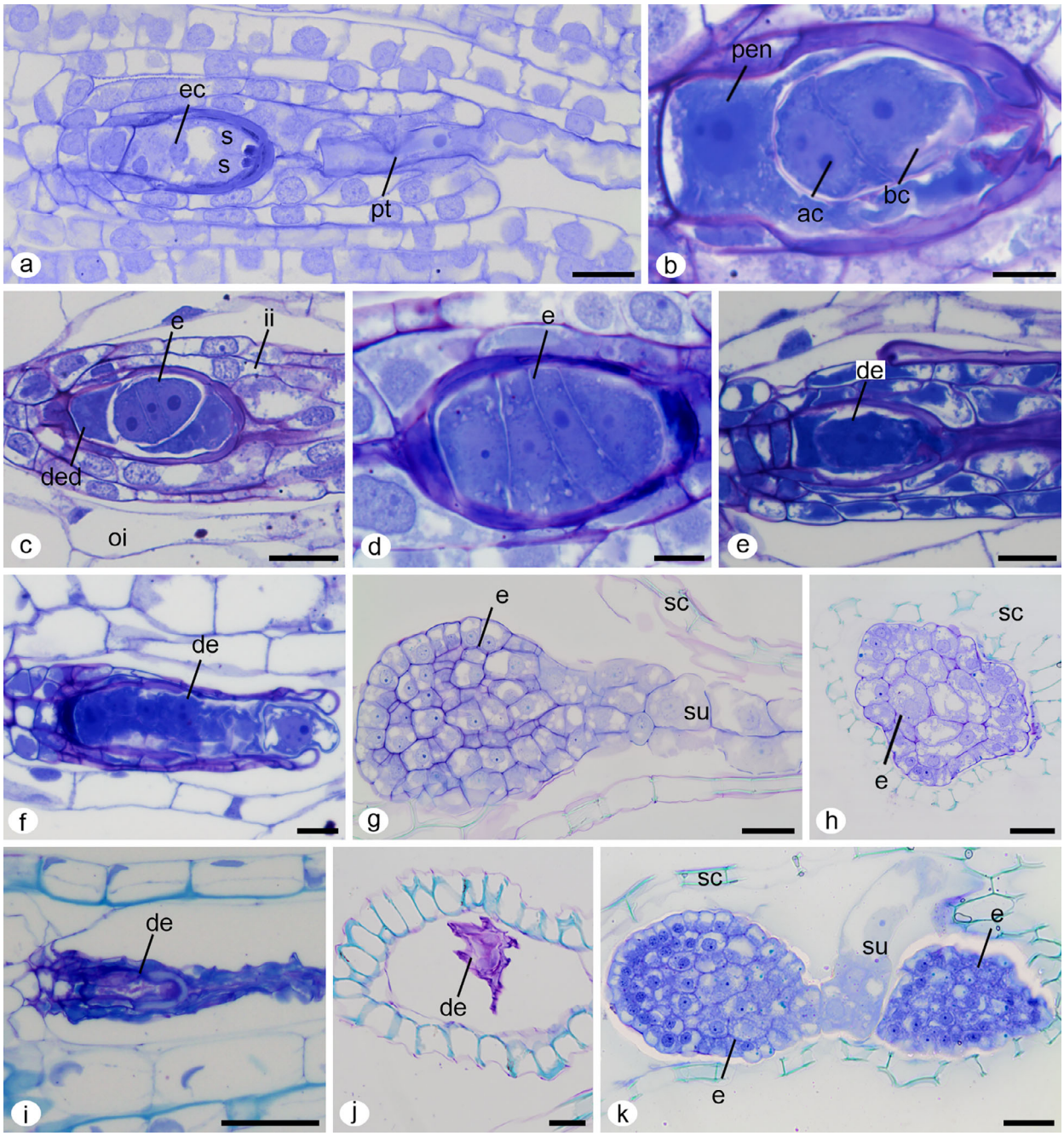

Fig. 5. Embryo and seed development of *Epidendrum* × *purpureum*. (a) Pollen tube reaching the female gametophyte. (b) Bicellular embryo, showing apical and basal cell, and primary endosperm nucleus. (c) Three-celled embryo. (d) Four-celled embryo. (e, f) Degenerating embryo. (g) Embryo with suspensor. (h) Embryo with irregular morphology. (i, j) Seed with degenerated embryo. (k) Seed with two embryos. ac, apical cell; bc, basal cell; e, embryo; ec, egg cell; de, degenerating embryo; ded, degenerating endosperm; ii, inner integument; oi, outer integument; pen, primary endosperm nucleus; pt, pollen tube; s, synergid; sc, seed coat; su, suspensor. Scale bars: a, c, e, f, j = 20 μm; b, d = 10 μm; g, h, i, j, k = 50 μm.

In most microspores, microgametogenesis occurs regularly, forming viable male gametophytes, which have vegetative and generative cells similar to those described for *E. orchidiflorum* and *E. denticulatum* (Alves *et al.* 2024). However, symmetrical mitotic divisions can be observed in the same pollinia, resulting in a male gametophyte with two identical cells, similar to the vegetative cells of functional male gametophytes. Studies have shown that asymmetric division for the formation of male gametophytes is essential and can be seen as a determining division for the function of the cells formed (Eady *et al.* 1995; Twell *et al.* 1998; Hackenberg & Twell 2019). In *Arabidopsis thaliana* (L.) Heynh. mutants and transgenic plants, symmetrical division during microgametogenesis is related to the loss of microspore polarity, the position of microtubules along the division, and failure during cytokinesis (Eady *et al.* 1995; Hackenberg & Twell 2019). In *E.* × *purpureum*, the formation of

symmetrical male gametophytes may be related to these events; however, further ultrastructural studies are required to clarify this issue.

In Orchidaceae, pollinia can be accompanied by different accessory structures (such as the caudicle, stipe, and viscidia) forming a unit called a pollinarium (Dressler 1993). As in pairs of parental species (Alves *et al.* 2024), caudicle development is observed in the hybrid *E.* × *purpureum*. Caudicles are characteristic structures of Orchidaceae that adhere pollen to the body of the pollinator and are considered the only example of haploid tissues with a non-sexual function (Johnson & Edwards 2000). The development of this accessory structure in the hybrid occurs in a way similar to that described for different species of the genus (Blackman & Yeung 1983; Johnson & Edwards 2000; Alves *et al.* 2024), with tissue differentiation and the formation of male gametophytes characterizing the caudicle as appendicular (Freudenstein & Rasmussen 1997).

Female gametophyte and embryo development in *E.* × *purpureum*

The hybrid *E.* × *purpureum* has flowers at anthesis with an undifferentiated ovary and the development of the placenta and ovule conditioned by the pollination process, similar to most orchids studied (Mayer *et al.* 2021). A few abnormalities were observed during female gametophyte formation. Although some ovules showed degeneration during megasporogenesis, in most cases the process occurs regularly, forming dyads and triads of megaspores, similar to the pairs of parental species. Megagametogenesis proceeds without alterations, with most ovules presenting viable female gametophytes with seven cells and eight nuclei at the end of development, similar to that observed in the parental species and in other species of this family (Sood 1986; Sood & Rao 1986; Mayer *et al.* 2011).

The formation of viable female gametophytes in *E.* × *purpureum* differs from that observed in the other hybrid specimens. In hybrids of *Oryza indica* L. (Poaceae) several abnormalities have been recorded. These include abnormal positioning of the nuclei of the female gametophyte, asynchronous nuclear migration, and the presence of a dark nucellus, resulting in degeneration of the female gametophyte (Zeng *et al.* 2009). Unlike the pattern observed in the parental species, in some ovules of *E.* × *purpureum* we observed the development of multiple archesporia, resulting in more than one female gametophyte per ovule. Multiple archesporia events are recognized as giving rise to more than one megaspore mother cell, which will develop sister tetrads that originate two reduced female gametophytes in a single ovule (e.g., Carmo-Oliveira *et al.* 2020). This event in Orchidaceae seems to be uncommon and has only been described for *Lecanorchis japonica* Blume and *Zygopetalum mackayi* Hook. (Johri *et al.* 1992; Costa *et al.* 2024).

After pollination, the pollen tube grows along the column without any interruption, confirming that the prezygotic reproductive barriers are weak. After fertilization, there is the formation of a zygote and a primary endosperm nucleus that soon degenerates, which are characteristics observed in different species of Orchidaceae (Yeung 2022). Zygotes underwent division to form an undifferentiated embryo and suspensor, similar to that described for the parental species and other species in the family (Yeung 2022). Eventually, we observed seeds with polyembryony resulting from ovules with multiple archesporia.

Despite the formation of viable embryos, most fertilized ovules showed degeneration. This event was expected and confirms that observed by Arida *et al.* (2021), who found low viability of seeds from self-pollination and cross-pollination between specimens of *E.* × *purpureum*. Low seed viability resulting from crosses between hybrids is common in several hybrid zones (Baack *et al.* 2015) and is often related to the occurrence of parental conflict during seed development. In many cases, seed nonviability is caused by a defective endosperm because of a deviation from the *2 m:1p* ratio, resulting in endosperm nonviability and consequent embryo degeneration (Köhler *et al.* 2021; Coughlan 2023). However, the absence of the endosperm in *E.* × *purpureum* is not directly related to low seed viability, as Orchidaceae endosperm degeneration is a common feature (Yeung 2017). Thus, embryo mortality in the seeds of this hybrid may be related to other genomic factors and may even be the result of the interaction of different genetic incompatibilities proposed in the Dobzhansky–Muller model, which predicts that hybrid collapse is caused by two or more mutational differences (Fishman & Sweigart 2018).

Backcrossing between hybrids and parental species: Fertilization and embryo development

Differences in ploidy and genome size between the parental species (*E. denticulatum* 2n = 52, *E. orchidiflorum* 2n = 156) and the hybrid (*E.* × *purpureum* 2n = 104 to 2n = 106) (Arida *et al.* 2021) did not appear to be a strong chromosomal barrier in the analysed hybrid zone. Regardless of whether hybrids received or donated pollen, no prezygotic incompatibility (e.g., pollen and stigma interactions and/or pollen tube and column interactions) occurred, indicating a high degree of interspecific compatibility between hybrids and parental species. According to Arida *et al.* (2021), prezygotic barriers such as habitat preference may play a stronger role in preventing the collapse of parental species in this hybrid zone.

Embryonic development was similar in all backcrosses, with the formation of an undifferentiated embryo, a suspensor with elongated cells, and a primary endosperm nucleus that soon degenerates. These characteristics are similar to those of the embryonic development of the parental species and species of different orchid genera (Yeung 2022). Embryo degeneration occurs in some backcrosses between hybrids and parents; as in embryos resulting from crosses between hybrids, embryo degeneration may be related to the accumulation of different genetic mechanisms of late incompatibility (Fishman & Sweigart 2018). Considering that not all embryos degenerate, and thousands of seeds are produced in each fruit, sporadic events of introgression cannot be ruled out for the hybrid zone studied here.

CONCLUSION

In this study, we analysed the role of different mechanisms involved in postzygotic isolation in the hybrid zone formed by pairs of the parental species, *E. denticulatum* and *E. orchidiflorum*, and the hybrid *E.* × *purpureum*. Our results show that the hybrid specimens present mostly viable gametophytes and gametes. After crosses between hybrids, most of the embryos

degenerated, a factor probably related to the accumulation of late genetic incompatibilities, confirming that although the individuals produce fertile gametes, the reproduction of the hybrids is compromised. Despite the differences in chromosome numbers between hybrids and parental species, the interaction between pollen and stigma occurred without any alteration, and the sterility of the seeds in this crossing resulted from genetic incompatibility. Further research is required to clarify the genetic and structural interactions related to the reduced fertility of this interspecific hybrid.

AUTHOR CONTRIBUTIONS

MFA and FP planned and designed the research. MFA and DSG performed the experimental pollinations and anatomical analyses. KLGT conducted confocal microscopy analysis. MFA wrote the manuscript. FP, DSG, KLGT and JFAB carefully reviewed the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Caudicle development in *Epidendrum* × *purpureum*. (a) Detail of the caudicle in the thecae of the anther. (b) Caudicle presenting two types of cells. Arrowheads indicate differentiating cells. (c) Cellulose in caudicle cell wall, evidenced by calcofluor. (d) Caudicle cells and sporangium cells of pollinia in meiosis. (e, f) Caudicle cell in meiosis indicated by arrowheads. (g) Overview of the caudicle after meiosis. (h) Caudicle cells showing two cells, vegetative and generative. (i) Deposition of sporopollenin in caudicle cells, evidenced by auramine. c, i Laser scanning confocal microscopy. ca, caudicle; ex, exine; gc, generative cell; mimc, microspore mother cell; ta, tapetum; vc, vegetative cell. Scale bars: a, b = 20 μm; c, d, g, i = 50 μm; e, f, h = 10 μm.

Fig. S2. Ovules and embryo development in the experimental treatment hybrid pollen receptor × parental pollen donor. a, d, e, f, g, j, n *Epidendrum denticulatum* pollen donor. b, c, h, i, j, k, l, o *E. orchidiflorum* pollen donor. (a) Overview of the ovary showing placental region with ovule primordia. (b) Differentiation of the initial archesporial cell and formation of the integuments (arrowheads). (c) Megaspore mother cell in meiosis I. (d) Megaspore dyad. (e) Functional chalazal megaspore expanding and degenerating megaspores. (f) Binucleated female gametophyte. (g) Tetranucleated female gametophyte with three nuclei in mitosis. (h) Female gametophyte with synergids, egg cell, two polar nuclei and antipode. (i) Penetrated synergid, zygote and polar nuclei. (j) Zygote evidencing the first mitotic division, and the primary endosperm nucleus. (k) Embryo with four cells. (l) Embryo and suspensor. (m) Degenerating embryo. (n, o) Unviable seed. a, antipodes; ac, archesporial cell; de, degenerating embryo; ded, degenerating endosperm; dm, degenerating megaspores; e, embryo; ec, egg cell; fm, functional megaspores; ii, inner integument; mmc, megaspore mother cell; ms, megaspores; nu, nucleus; oi, outer integument; op, ovule primordium; p, placental region; pen, primary endosperm nucleus; pn, polar nuclei; ps, penetrated synergid; s, synergids; sc, seed coat; su, suspensor. Scale bars a = 200 μm; b, c, d, e, f, g, h, i, j, m, n = 20; k = 10 μm; l, o = 50 μm.

Fig. S3. Ovules and embryo development in the experimental treatment parental pollen receptor × hybrid pollen donor. a, b, f, h, j, l, o *Epidendrum denticulatum* pollen receptor. c, d, e, g, i, k, m, n *E. orchidiflorum* pollen receptor. (a) Overview of undifferentiated ovary at anthesis. (b) Overview of the ovary showing sterile valve and fertile valve. (c) Detail of placental region showing ovule primordium. (d) Differentiation of the initial archesporial cell and formation of the integuments (arrowheads). (e) Megaspore mother cell. (f) Megaspores dyad. (g) Functional chalazal megaspore expanding and degenerating megaspores. (h) Binucleated female gametophyte. (i) Two female gametophytes in the same ovule. (j) Tetranucleated female gametophyte. (k) Female gametophyte with synergids, egg cell, and polar nucleus. (l) Penetrated synergid and zygote. (m) Embryo and suspensor. (n) Degenerating embryo. (o) Unviable seeds. ac, archesporial cell; de, degenerating embryo; dm, degenerating megaspores; e, embryo; ec, egg cell; fm, functional megaspores; fv, fertile valve; ii, inner integument; mmc, megaspore mother cell; ms, megaspores; nu, nucleus; oi, outer integument; op, ovule primordium; p, placental region; pn, polar nuclei; ps, penetrated synergid; s, synergids; sc, seed coat; sv, sterile valve; su, suspensor. Scale bars: a, b=. 200 μm; c = 50 μm; d, e, i, k = 10 μm; f, g, h, j, l, m, n, o = 20 μm.

Fig. S4. Ovule and embryo development of *Epidendrum denticulatum*. (a) Overview of the ovary showing sterile valve and fertile valve. (b) Placental regions with ovules in the early stages of development. (c) Differentiation of the initial archesporial cell and formation of integuments indicated by arrowheads. (d) Megaspore mother cell during meiosis I. (e) Megaspores dyad. (f) Functional chalazal megaspore expanding and degenerating megaspores. (g) Binucleated female gametophyte. (h) Female gametophyte with three visible nuclei. (i) Female gametophyte with synergids, egg cell and antipodes. (j) Penetrated synergids. (k). Zygote. (l) Embryo with three cells. (m) Embryo with four cells. (n) Embryo with evident suspensor. ac, archesporial cell; de, degenerating embryo; dm, degenerating megaspores; e, embryo; ec, egg cell; fm, functional megaspores; fv, fertile valve; mmc, megaspore mother cell; ms, megaspores; ne, nucelar epidermis; nu, nucleus; op, ovule primordium; p, placental region; ps, penetrated synergid; s, synergids; sc, seed coat; sv, sterile valve; su, suspensor. Scale bars: a = 200 μm; b, n = 100 μm; c, d, e, f, g, h = 20 μm; i, k, l, m = 10 μm; j = 50 μm.

Fig. S5. Ovule and embryo development of *Epidendrum orchidiflorum*. (a) Overview of the ovary showing sterile valve and fertile valve. (b) Placental regions with ovules in the early stages of development. (c) Differentiation of the initial archesporial cell. Black arrows indicate integument differentiation. (d) Megaspore mother cell. (e) Megaspore dyad. (f) Functional chalazal megaspore. (g) Binucleated female gametophyte. (h) Female gametophyte with three visible nuclei. (i) Female gametophyte. (j) Penetrated synergids and egg cell. (k) Embryo showing apical cell and basal cell. (l). Three-celled proembryo. (m) proembryo and suspensor. (n) Embryo, with evident suspensor. ac, archesporial cell; de, degenerating embryo; dm, degenerating megaspores; e, embryo; ec, egg cell; fm, functional megaspores; fv, fertile valve; mmc, megaspore mother cell; ms, megaspores; ne, nucelar epidermis; nu, nucleus; op, ovule primordium; p, placental region; ps, penetrated synergid; s, synergids; sc, seed coat; sv, sterile valve; su, suspensor. Scale bars: a = 200 μm; b = 100 μm; c = 20 μm; d, e, f, g, h, I, j, k, l, m, n = 50 μm.

REFERENCES

Abbott R. (2017) Plant speciation across environmental gradients and the occurrence and nature of hybrid zones. *Journal of Systematics and Evolution*, **55**, 238–258.

Alves M.F., Pinheiro F., De Toni K.L.G., Baumgratz J.F.A. (2024) Anatomical features of pollinia and caudicle in *Epidendrum* (Orchidaceae; Epidendroideae). *Brazilian Journal of Botany*, **47**, 219–228.

Arida B.L., Scopece G., Machado R.M., Moraes A.P., Fornini-Martins E., Pinheiro F. (2021) Reproductive barriers and fertility of two neotropical orchid species and their natural hybrid. *Evolutionary Ecology*, **35**, 41–64.

Azevedo C.O., Borba E.L., Van Der Berg C. (2006) Evidence of natural hybridization and introgression in *Bulbophyllum involutum* Borba, Semir & F. Barros and *B. Weddellii* (Lindl.) Rchb. f. (Orchidaceae) in the Chapada Diamantina, Brazil, by using allozyme markers. *Revista Brasileira de Botânica*, **29**, 415–421.

Baack E., Melo M.C., Rieseberg L.H., Ortiz-Barrientos D. (2015) The origins of reproductive isolation in plants. *New Phytologist*, **207**, 968–984.

Bhandari N.N. (1984) The microsporangium. In: Johri B.M. (Ed), *Embryology of angiosperms*. Springer, Berlin, Germany, pp 53–122.

Blackman S., Yeung E.C. (1983) Structural development of the caudicle of an orchid (*Epidendrum*). *American Journal of Botany*, **70**, 97–105.

Borba E.L., Semir J. (1998) *Bulbophyllum* x*cipoense* (Orchidaceae), a new natural hybrid from the Brazilian "campos rupestres": Description and biology. *Lindleyana*, **13**, 113–120.

Carmo-Oliveira R., Custodio L.N., De Morretes B.L., Oliveira P.E. (2020) Early embryology of Vochysiaceae and some insights into its phylogeny and intrafamilial taxonomy. *Phytotaxa*, **443**, 211–257.

Coleman A.W., Golf L.J. (1985) Applications of fluorochromes to pollen biology. I. Mithramycin and 4′,6-diamidino-2-phenylindole (DAPI) as vital stains for quantitation of nuclear DNA. *Stain Technology*, **60**, 145–154.

Costa G.V., Alves M.F., Duarte M.O., Caetano A.P.S., Koehler S., Mayer J.L.S. (2024) Apomixis beyond trees in the Brazilian savanna: New insights from the orchid *Zygopetalum mackayi*. *AoB Plants*, **16**, plae037.

Coughlan J.M. (2023) The role of conflict in shaping plant biodiversity. *New Phytologist*, **240**, 2210–2217.

Coyne J., Orr H. (2004) *Speciation: A catalogue and critique of species concepts*. Sinauer Associates, Sunderland, MA, USA, pp 533.

Cozzolino S., Scopece G. (2008) Specificity in pollination and consequences for reproductive isolation in deceptive Mediterranean orchids. *Philosophical Transitions of the Royal Society*, **363**, 3037–3046.

Darwin C. (1859) *On the origin of species by means of natural selection, or the preservation of favoured races in the struggle for life*. John Murray, London, UK, pp 516.

de Queiroz K. (2007) Species concepts and species delimitation. *Systematic Biology*, **56**, 879–886.

Dobzhansky T. (1937) *Genetics and the origin of species*. Columbia University Press, New York, pp 364.

Dressler R.L. (1993) *Phylogeny and classification of the orchid family*. Dioscorides Press, Portland, pp 330.

Eady C., Lindsey K., Twell D. (1995) The significance of microspore division and division symmetry for vegetative cell-specific transcription and generative cell differentiation. *The Plant Cell*, **7**, 65–74.

Fiorini C.F., Smidt E.C., Knowles L.L., Borba E.L. (2023) Hybridization boosters diversification in a neotropical *Bulbophyllum* (Orchidaceae) group. *Molecular Phylogenetics and Evolution*, **186**, 107858.

Fishman L., Sweigart A.L. (2018) When two rights make a wrong: The evolutionary genetics of plant hybrid incompatibilities. *Annual Review of Plant Biology*, **69**, 707–731.

Freudenstein J.V., Rasmussen F.N. (1997) Sectile pollinia and relationships in the Orchidaceae. *Plant Systematics and Evolution*, **205**, 125–146.

Gautam R., Shukla P., Kirti P.B. (2023) Male sterility in plants: An overview of advancements from natural CMS to genetically manipulated systems for hybrid seed production. *Theoretical and Applied Genetics*, **136**, 195.

Gerrits P.O., Smid L. (1983) A new less toxic polymerization system for the embedding of soft tissues in glycol methacrylate and subsequent preparing of serial sections. *Journal of Microscopy*, **132**, 81–85.

Ghimire B., Son S., Kim J.H., Jeong M.J. (2020) Gametophyte and embryonic ontogeny: Understanding the reproductive calendar of *Cypripedium japonicum* Thunb. (Cypripedoideae, Orchidaceae), a lady's slipper orchid endemic to East Asia. *BMC Plant Biology*, **20**, 1–14.

Hackenberg D., Twell D. (2019) The evolution and patterning of male gametophyte development. *Current Topics in Developmental Biology*, **131**, 257–298.

Hafidh S., Fíla J., Honys D. (2016) Male gametophyte development and function in angiosperms: A general concept. *Plant Reproduction*, **29**, 31–51.

Hágsater E., Soto-Arenas M.A. (2005) Epidendrum. In: Pridgeon A.M., Cribb P.J., Chase M.W., Rasmussen F.N. (Eds), *Genera Orchidacearum Epidendroideae (part 1)*, Vol. **4**. Oxford University Press, Oxford, UK, pp 236–238.

Heslop-Harrison Y. (1977) The pollen-stigma interaction: Pollen-tube penetration in *Crocus*. *Annals of Botany*, **41**, 913–922.

Johnson S.D., Edwards T.J. (2000) The structure and function of orchid pollinaria. *Plant Systematics and Evolution*, **222**, 243–269.

Johri B.M., Ambegaokar K.B., Srivastava P.S. (1992) *Comparative embryology of angiosperms*. Springer, Berlin, Germany, pp 1221.

Kant R., Goel S. (2013) Development of anther in *Goodyera repens* (L.) R. Br a Massulate Orchid. *Vegetos*, **26**, 9–18.

Köhler C., Dziasek K., Del Toro-De L.G. (2021) Post-zygotic reproductive isolation established in the endosperm: Mechanisms, drivers and relevance. *Philosophical Transactions of the Royal Society B*, **376**, 20200118.

Lillie R.D. (1965) *Histopathologic technic and practical histochemistry*. McGraw Hill, New York, USA, pp 942.

Marques I., Draper D., Riofrío L., Naranjo C. (2014) Multiple hybridization events, polyploidy and low postmating isolation entangle the evolution of neotropical species of *Epidendrum* (Orchidaceae). *Evolutionary Biology*, **14**, 20–34.

Mayer E.S., Winiarczyk K., Błaszczyk L., Kosmala A., Rabinowitch H.D., Kamenetsky R. (2013) Male gametogenesis and sterility in garlic (*Allium sativum* L.): Barriers on the way to fertilization and seed production. *Planta*, **237**, 103–120.

Mayer J.L.S., Carmello-Guerreiro S.M., Appezzato-da-Glória B. (2011) Anatomical development of the pericarp and seed of *Oncidium flexuosum* Sims (Orchidaceae). *Flora*, **206**, 601–609.

Mayer J.L.S., Scopece G., Lumaga M.R.B., Coiro M., Pinheiro F., Cozzolino S. (2021) Ecological and phylogenetic constraints determine the stage of anthetic ovule development in orchids. *American Journal of Botany*, **108**, 2405–2415.

Mayr E. (1942) *Systematics and the origin of species*. Columbia University Press, New York, pp 382.

Moraes A.P., Chinaglia M., Palma-Silva C., Pinheiro F. (2013) Interploidy hybridization in sympatric zones: The formation of *Epidendrum fulgens* × *E. puniceoluteum* hybrids (Epidendroideae, Orchidaceae). *Ecology and Evolution*, **3**, 3824–3837.

Moyle L.C., Jewell C.P., Kostyun J.L. (2014) Fertile approaches to dissecting mechanisms of premating and postmating prezygotic reproductive isolation. *Current Opinion in Plant Biology*, **18**, 16–23.

O'Brien T.P., McCully M.E. (1981) *The study of plant structure: Principles and selected methods*. Termacarphi, Melbourne, Australia, pp 357.

Oparka K.J., Read N.D. (1994) The use of fluorescent probes for studies of living plant cells. In: Harris N., Oparka K.J. (Eds), *Plant cell biology: A practical approach*. Oxford University Press, Oxford, UK, pp 120–132.

Pachon A.M.R. (2016) Filogeografa a partir de DNA de cloroplasto da orquídea neotropical *Epidendrum orchidiforum* (Orchidaceae: Laeliinae) no Brasil. Ph.D. Dissertation. Universidade de São Paulo, Brazil.

Pellegrino G., Bellusci F., Musacchio A. (2010) Strong post-pollination pre-zygotic isolation between sympatric, food-deceptive Mediterranean orchids. *Sexual Plant Reproduction*, **23**, 281–289.

Pinheiro F., Barros F., Palma-Silva C., Meyer D., Fay M.F., Suzuki R.M., Lexer C., Cozzolino S. (2010) Hybridization and introgression across different ploidy levels in the neotropical orchids *Epidendrum fulgens* and *E. Puniceoluteum* (Orchidaceae). *Molecular Ecology*, **19**, 3981–3994.

Pinheiro F., Cardoso-Gustavson P., Suzuki R.M., Abrao M.C.R., Guimarães L.R.S., Draper D., Moraes A.P. (2015) Strong postzygotic isolation prevents introgression between two hybridizing neotropical orchids, *Epidendrum denticulatum* and *E. Fulgens*. *Evolutionary Ecology*, **29**, 229–248.

Pinheiro F., Cozzolino S. (2013) *Epidendrum* (Orchidaceae) as a model system for ecological and evolutionary studies in the neotropics. *Taxon*, **62**, 77–88.

Pinheiro F., Gouveia T.M.Z., Cozzolino S., Cafasso D., Cardoso-Gustavson P., Suzuki R.M., Palma-Silva C. (2016) Strong but permeable barriers to gene exchange between sister species of *Epidendrum*. *American Journal of Botany*, **103**, 1472–1482.

Rodrigues B.J. (1877) Genera et species Orchidearum Novarum v.1. Typographia Nacional, Sebastianópolis (p. 316).

Sakai W.S. (1973) Simple method for differential staining of paraffin embedded plant material using toluidine blue O. *Stain Technology*, **48**, 247–249.

Scopece G., Croce A., Lexer L., Cozzolino S. (2013) Components of reproductive isolation between *Orchis mascula* and *Orchis pauciflora*. *Evolution*, **67**, 2083–2093.

Scopece G., Widmer A., Cozzolino S. (2008) Evolution of postzygotic reproductive isolation in a guild of deceptive orchids. *American Society of Naturalists*, **171**, 315–326.

Shin H., Park H.R., Park J.E., Yu S.H., Yi G., Kim J.H., Koh W., Kim H.H., Lee S.S., Huh J.H. (2021) Reduced fertility caused by meiotic defects and micronuclei formation during microsporogenesis in × *Brassicoraphanus*. *Genes & Genomics*, **43**, 251–258.

Sobel J.M., Chen G.F., Watt L.R., Schemske D.W. (2010) The biology of speciation. *Evolution*, **64**, 295–315.

Sood S.K. (1986) Gametogenesis, integuments initiation and embryogeny in three species of *Habenaria* (Orchidaceae, Orchideae). *Proceedings of the Indian National Science Academy*, **96**, 487–494.

Sood S.K., Rao P.R.M. (1986) Gametophytes, embryogeny and pericarp of *Microstylis wallichii* Lindl. (Orchidaceae). *Botanical Magazine*, **99**, 351–359.

Sood S.K., Rao P.R.M. (1988) Studies in the embryology of the diandrous orchid *Cypripedium cordigerum* (Cypripedioideae, Orchidaceae). *Plant Systematics and Evolution*, **160**, 159–168.

Swamy B.G.L. (1949) Embryological studies in the Orchidaceae. II. Embryogeny. *American Midland Naturalist*, **41**, 202.

Szlachetko D.L., Kolanowska M., Muller F., Vannini J., Rojek J., Górniak M. (2017) First Guatemalan record of natural hybridization between neotropical species of the Lady's slipper orchid (Orchidaceae, Cypripedioideae). *PeerJ*, **225**, e4162.

Tao Z.B., Ren Z.X., Bernhardt P., Liang H., Li H.D., Zhao Y.H., Wang H., Li D. (2018) Does reproductive isolation reflect the segregation of color forms in *Spiranthes sinensis* (pers.) Ames complex (Orchidaceae) in the Chinese Himalayas? *Ecology and Evolution*, **8**, 5455–5469.

Twell D., Park S.K., Lalanne E. (1998) Asymmetric division and cell-fate determination in developing pollen. *Trends in Plant Science*, **3**, 305–310.

Vega Y., Marques I., Castro S., Loureiro J. (2013) Outcomes of extensive hybridization and introgression in *Epidendrum* (Orchidaceae): Can we rely on species boundaries? *PLoS One*, **8**, 1–13.

Whitehead M.R., Peakall R. (2014) Pollinator specificity drives strong prepollination reproductive isolation in sympatric sexually deceptive orchids. *Evolution*, **68**, 1561–1575.

Widmer A., Lexer C., Cozzolino S. (2009) Evolution of reproductive isolation in plants. *Heredity*, **102**, 31–38.

Xu S.Q., Schluter P.M., Scopece G., Breitkopf H., Gross K., Cozzolino S., Schiestl F.P. (2011) Floral isolation is the main reproductive barrier among closely related sexually deceptive orchids. *Evolution*, **65**, 2606–2620.

Yeung E.C. (2017) A perspective on orchid seed and protocorm development. *Botanical Studies*, **58**, 1–14.

Yeung E.C. (2022) The orchid embryo – "An embryonic protocorm". *Botany*, **100**, 691–706.

Zeng Y.X., Hu C.Y., Lu Y.G., Li J.Q., Liu D.X. (2009) Abnormalities occurring during female gametophyte development result in the diversity of abnormal embryo sacs and leads to abnormal fertilization in *indica/japonica* hybrids in rice. *Journal of Integrative Plant Biology*, **51**, 3.

Zhang H.P., Tao Z.B., Trunschke J., Shrestha M., Scaccabarozzi D., Wang H., Ren Z.X. (2022) Reproductive isolation among three nocturnal moth-pollinated sympatric *Habenaria* species (Orchidaceae). *Frontiers in Plant Science*, **13**, 908852.

Zhang L., Ma M., Cui L., Liu L. (2021) Deciphering the dynamic gene expression patterns of pollen abortion in a male sterile line of *Avena sativa* through transcriptome analysis at different developmental stages. *BMC Plant Biology*, **21**, 101.